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in a Conditional Transgenic Model

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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	11
Reportable Outcomes.....	12
Conclusions.....	12
References.....	12
Appendices.....	13

INTRODUCTION:

As described in the 2001 annual report, the focus of this research has shifted from a study of conditional expression of c-Myc in the mammary gland to the conditional expression of three other oncogenes. The pathways stimulated by the oncogenes HER2/Neu/ErbB2, Wnt-1, and H-ras are aberrantly activated in many human cancers, including breast cancer, and have been shown to play a role in carcinogenesis. In order to better investigate the role these genes play in breast cancer, we generated mouse models that inducibly express activated forms of these oncogenes in the mammary gland. This system allows us to activate these oncogenic pathways during specific windows of mammary gland development, as well as to remove the initiating oncogenic stimulus after the development of a tumor or during later stages of tumor progression. As such, we anticipate that the study of these models will provide valuable insights into the mechanisms by which activation of these pathways contributes to breast cancer development or progression, and will ultimately aid in the generation of therapeutic strategies that specifically target these pathways.

BODY:

Technical Objective 1, Task 1: Months 1-12: Cloning and injection of constructs containing each oncogene under the control of the tetop promoter to establish founder lines.

Constructs for transgenic injection were generated by cloning the coding sequence of activated Neu, activated H-ras (v-H-ras), or Wnt-1 after the Tet operator/minimal CMV promoter (pTetSplice). An internal ribosomal entry sequence (IRES) followed by the firefly luciferase coding sequence were cloned downstream of the oncogene for use as a reporter of transgene expression. Constructs were injected into fertilized embryos at the one- or two-cell stage by the Transgenic Core facility. Tail DNA from founder mice was screened by PCR to determine the presence of a transgene. Fourteen founders were identified from the Tetop-Activated Neu (TAN) transgene, eight from the Tetop-Wnt-1 (TWNT), and two from the Tetop-v-H-ras transgene. Southern analysis of each of these founders indicated that each transgene had integrated at only one genomic locus.

Technical Objective 1, Task 2: Months 1-12: Testing of founder mice for germline transgene transmission.

Each mouse identified as a founder was bred to a wild-type mouse to test for germline transmission of the transgene. Three TAN founders, four TWNT founders, and one TRAS founder bred successfully and were found to transmit the transgene to their offspring.

Technical Objective 1, Task 3: Months 6-12: Testing of founder lines for transgene expression and inducibility.

In order to test for transgene expression, each founder that carried the transgene in its germline was bred to the MMTV-rtTA (MTB) line described in this proposal. Female bitransgenic mice that bore both MTB (thereby expressing rtTA in the mammary epithelium) and either TAN, TWNT, or TRAS were given doxycycline in their drinking water for four days. Mammary gland tissue was harvested from these animals, as well as from bitransgenic mice maintained off doxycycline, to examine transgene expression levels and inducibility by both luciferase assay and Northern analysis. All of the tested founder lines had undetectable levels of transgene RNA in the uninduced state, but displayed varying levels of transgene expression upon induction with doxycycline. This was true both at the RNA level and at the protein level, as measured by luciferase assay (Fig. 1 and Fig. 2). Thus, the founder line for each Tetop-oncogene that displayed the greatest level of transgene expression upon incuction was chosen for further analysis.

Technical Objective 1, Task 4: Months 12-24: Examination of histological phenotypes resulting as a consequence of short-term transgene expression.

We next examined the phenotypes resulting from short-term induction of each oncogene in the mammary gland. Bitransgenic female animals were induced with doxycycline for four days, at which time mammary glands were whole mounted, fixed in paraformaldehyde, and either carmine stained or blocked, sectioned, and stained with hematoxylin and eosin. Striking morphological and histological changes were evident in response to overexpression of each oncogene even after only four days. MTB/TAN animals develop a hyperplastic glandular phenotype throughout the mammary epithelium, while MTB/TWNT animals show a uniform increase in ductal branching. MTB/TRAS mice display a highly abnormal mammary gland phenotype, in many areas lacking a distinct glandular morphology (Fig. 3). Consistent with the RNA and protein data, mammary glands from uninduced bitransgenic females are phenotypically indistinguishable from those of wild-type animals.

Technical Objective 2, Task 1: Months 12-24: Testing of each oncogene line for mammary tumor development.

We established during year 1 that bitransgenic mice from each line—MMTV-rtTA/Tetop-NeuNT (MTB/TAN), MMTV-rtTA/Tetop-Wnt1 (MTB/TWNT), and MMTV-rtTA/Tetop-v-H-ras (MTB/TRAS)—expressed the oncogene in a doxycycline-dependent manner specifically within the mammary gland. We next sought to determine whether chronic induction of these animals with doxycycline would result in mammary tumors. Bitransgenic female mice were given 2 mg/ml doxycycline in their drinking water beginning at 6 weeks of age. They were inspected and palpated weekly for mammary tumor development. We found that all of the bitransgenic animals maintained on doxycycline developed mammary tumors, whereas neither uninduced bitransgenics nor any of the monotransgenics maintained on doxycycline for periods of over a year developed tumors (Fig. 4 and data not shown). Average tumor latencies were 6 wks for MTB/TAN and 20 wks for MTB/TWNT at 2 mg/ml doxycycline, and 13 wks for MTB/TRAS at 0.012 mg/ml doxycycline. MTB/TRAS animals were chronically induced at a lower dose because they rapidly become ill when induced at 2 mg/ml, a reaction that we speculate may be due to increased circulating cytokine levels stimulated by high transgene levels.

Having determined that all the lines provided functional tumor models, I focused on pursuing the MTB/TAN model, given the known importance of HER2/Neu in human breast cancers. We first wished to determine whether transgene expression levels could be modulated by titrating doses of doxycycline. We induced bitransgenic females at 0.1 mg/ml, 0.2 mg/ml, and 0.4 mg/ml doxycycline for 14 days (n=8-10 per group), beginning at 6 weeks of age, and harvested mammary gland tissue to determine transgene expression levels by Northern analysis. We found that the transgene was expressed in a doxycycline dose dependent manner (Fig. 5). We then chronically induced animals at these doses and monitored them weekly for tumor development. We found that they developed mammary gland tumors with complete penetrance and in a doxycycline dose-dependent manner (Fig. 6 and Table 1).

Technical Objective 2, Task 2: Months 12-24: Pathologic analysis of tumors or chronically treated tissues.

Tumors were harvested from chronically induced animals, fixed overnight in 4% paraformaldehyde, and blocked in paraffin. Histological analysis of the tumors revealed that they were invasive tumors, and they were pathologically classified as solid nodular carcinomas. Both the classically described MMTV-Neu tumor phenotype and a more atypical “swirling” pattern of epithelial and stromal cells were identifiable within individual tumors (Fig. 7). Cells within the tumors had large nuclei with an open chromatin pattern. Immunohistochemical analysis of these tumors confirmed abundant levels of Neu (Fig. 7).

Technical Objective 2, Task 3: Months 12-24: Examination of lungs and livers of tumor-bearing animals for evidence of metastases.

MTB/TAN animals that were maintained on doxycycline after initial tumor development invariably developed a distinctive respiratory distress phenotype, characterized by labored breathing, unkempt fur,

hunched posture, and diminished activity. Twenty-four of 26 mice sacrificed while bearing this phenotype were found to harbor grossly detectable pulmonary metastases. Histological analysis of these lungs confirmed the presence of Neu-type mammary epithelial tumor nodules embedded within the normal lung parenchyma (Fig. 8). Liver nodules have only been detected in one animal thus far.

Technical Objective 2, Task 4: Months 18-36: Examination of mammary gland or mammary tumor phenotype following doxycycline withdrawal.

Once mammary gland tumors had developed in MTB/TAN animals, we wished to determine whether these tumors remained dependent upon Neu transgene expression for maintenance of the transformed state. We removed animals with tumors from doxycycline treatment and observed that 44 of 47 tumors monitored regressed to a non-palpable state, indicating that the vast majority of cells within these tumors do require Neu overexpression to maintain the transformed state. To determine the cellular mechanism for tumor regression, we examined proliferation and apoptosis rates in tumors taken from animals 48 hours after doxycycline withdrawal. BrdU staining indicated that the high rate of proliferation present in tumors on doxycycline diminished dramatically within 48 hours of doxycycline withdrawal, while TUNEL staining demonstrated an increase in numbers of apoptotic cells in the regressing tumors (Fig. 9). Thus, it appears that the Neu-induced tumors regress as a result of both a decrease in proliferation and an increase in apoptosis.

Technical Objective 2, Task 5: Months 18-36: Oligonucleotide microarray analysis of short-term induction tissue to identify differences in pathways activated *in vivo* by each oncogene.

To begin to dissect the stages of mammary tumor development, we performed gene expression profiling using oligonucleotide microarrays. Beginning at 6 weeks of age MTB/TAN animals and MTB controls were treated with doxycycline for 0, 24, 48, and 96 hours, as well as 21 days. Each timepoint was analyzed in triplicate by microarray, with each sample composed of tissue pooled from three animals. Analyses of these data are ongoing.

Technical Objective 3, Task 1: Months 18-36: Generate tritransgenic animals carrying transgenes for MMTV-rtTA and different pairs of the four Tetop-oncogene (c-myc, wnt-1, v-H-ras, and activated Neu).

Since we had decided to focus on Neu tumor biology, we generated tritransgenic animals that carried the MMTV-rtTA and Tetop-activated Neu transgenes (MTB/TAN), as well as either Tetop-c-myc or Tetop-wnt-1 (MTB/TAN/TOM and MTB/TAN/TWNT, respectively). In the uninduced state, all of these animals were indistinguishable from wild-type controls.

Technical Objective 3, Task 2: Months 18-36: Morphological analysis of mammary gland tissue overexpressing two oncogenes as compared to each of the two overexpressed alone.

Tritransgenic animals and their bitransgenic controls were induced with 2 mg/ml doxycycline at 6 weeks of age. After 14 days animals were sacrificed and mammary glands were examined for gross morphology as well as for histology. We found that a more severely hyperproliferative phenotype was present in both MTB/TAN/TOM and MTB/TAN/TWNT mice than in MTB/TAN, MTB/TOM, or MTB/TWNT mice (Fig. 10 and 11 and data not shown).

Technical Objective 3, Task 3: Months 18-36: Chronic inductions of tritransgenic animals to determine differences in tumor latencies and/or metastatic potential.

MTB/TAN/TWNT, MTB/TAN/TOM, MTB/TAN, MTB/TWNT, and MTB/TOM mice were induced chronically with doxycycline at 0.1 mg/ml and examined weekly for tumor development. Both MTB/TAN/TOM and MTB/TAN/TWNT animals developed mammary tumors with a much shorter latency than MTB/TAN, MTB/TOM, or MTB/TWNT animals (Table 2, Fig. 12, and data not shown). No difference was noted in the incidence of pulmonary metastases in tumor-bearing animals.

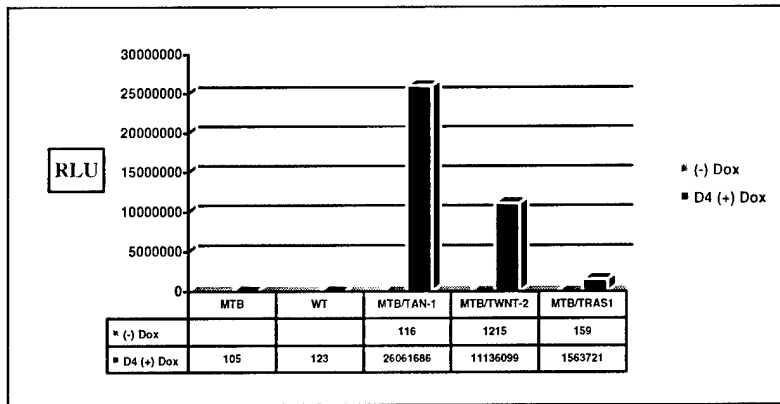


Fig. 1 Luciferase activity assays of MTB, WT, MTB/TAN, MTB/TWNT, and MTB/TRAS mammary glands in the presence and absence of doxycycline.

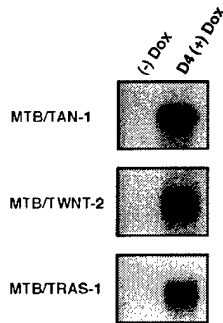


Fig. 2 Northern analysis of mammary gland total RNA from MTB/TAN, MTB/TWNT, and MTB/TRAS animals. Each Northern blot was hybridized with a cDNA probe specific for the indicated transgene.

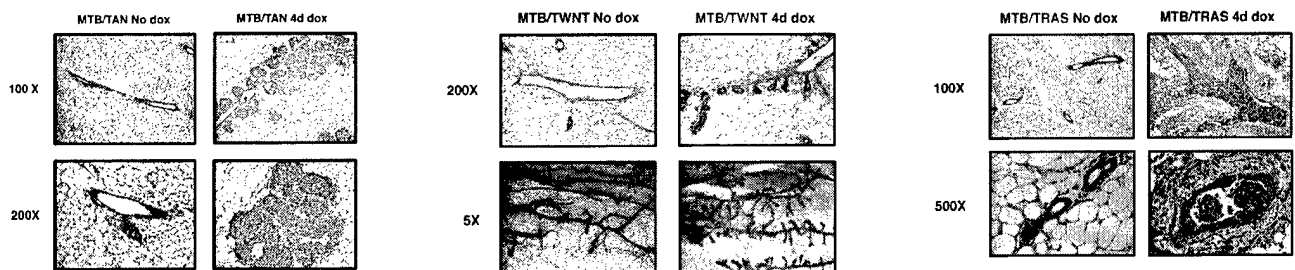


Fig. 3 Whole mounts and H&E stained sections of uninduced and 96h-induced MTB/TAN, MTB/TWNT, and MTB/TRAS mammary glands.

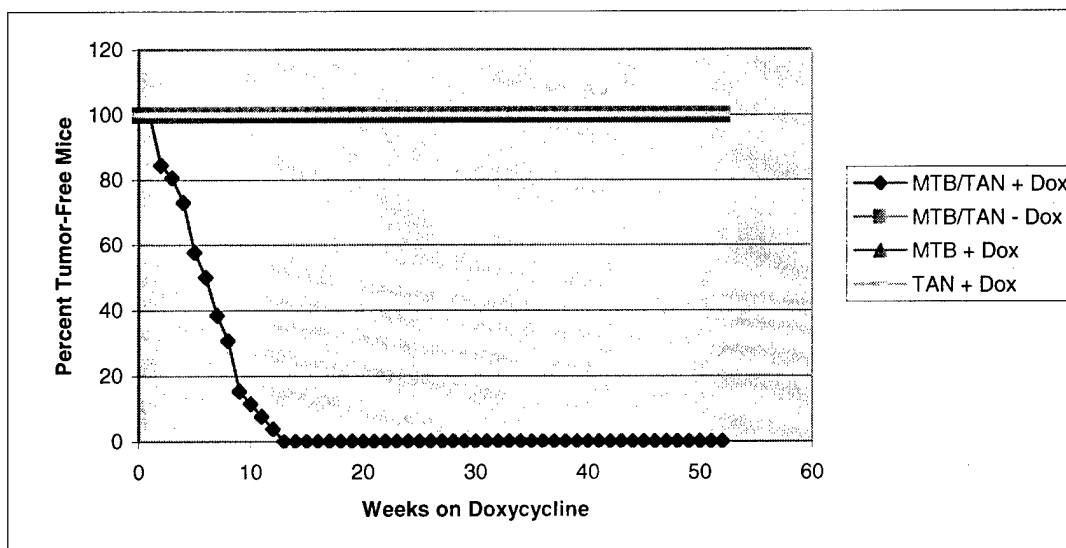


Fig. 4. Tumor-free survival curves of MTB/TAN animals and genetic controls on doxycycline, and of uninduced MTB/TAN animals.

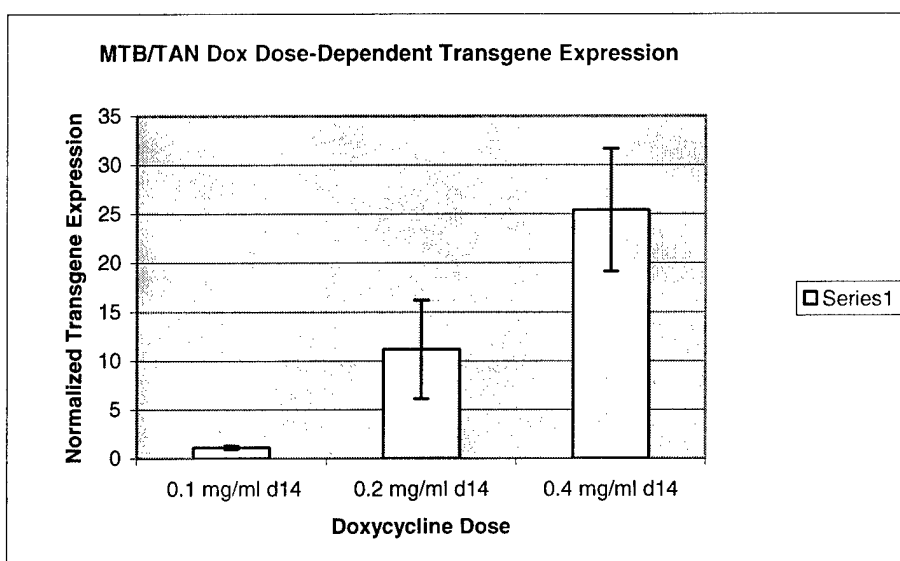


Fig. 5. Dose-dependent transgene expression in MTB/TAN animals. Phosphorimager quantitation of Northern analysis of MTB/TAN mammary glands from animals treated for 14 days with the indicated doses of doxycycline. TAN transgene levels were normalized to β -actin levels and averaged (8-10 animals analyzed per group).

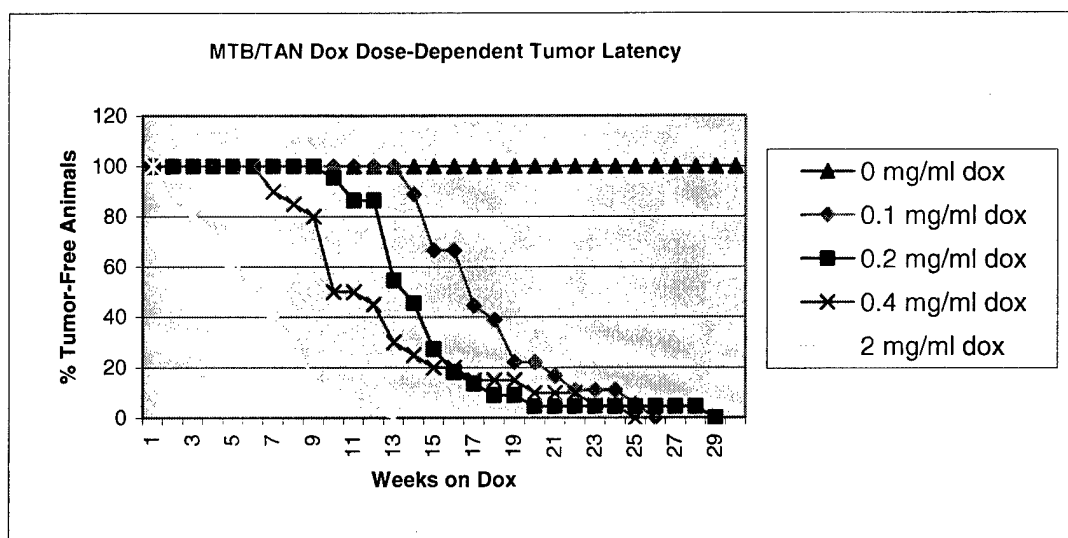


Fig. 6. Dose-dependent tumor latencies. Tumor-free survival curves from MTB/TAN animals treated chronically with each of the indicated doxycycline doses.

	Dox Dose (mg/ml)			
	0.1	0.2	0.4	2
Mean Latency (wks)	17.55	14.39	12.35	6.21
Std. Dev	3.52	3.93	5.06	3.10
SEM	0.83	0.84	1.13	0.61
N	18	22	20	26

Table 1. Dose-dependent tumor latencies. Average latency to detection of the first tumor in MTB/TAN animals induced with doxycycline at the indicated doses.

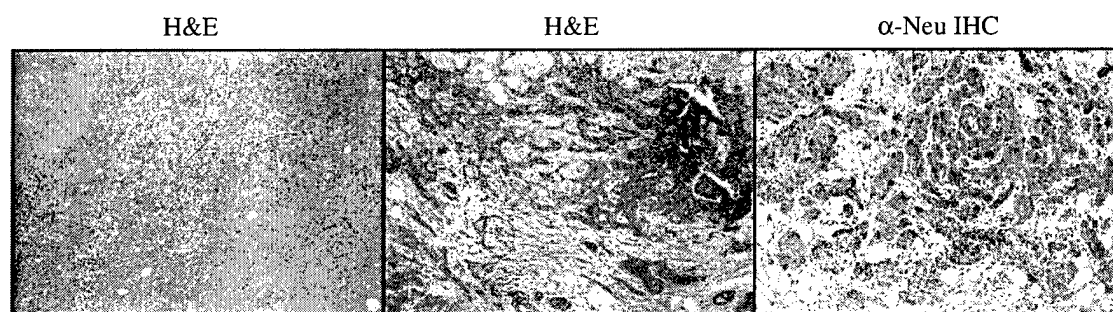


Fig. 7. Histology and immunohistochemistry of MTB/TAN tumors. Left panel, hematoxylin and eosin (H&E)-stained section displaying the classically described Neu tumor phenotype. Middle panel, H&E-stained section displaying a tumor region with a swirling pattern of epithelium and stroma. Right panel, α -Neu immunohistochemistry confirming the presence of high levels of Neu throughout these tumors.

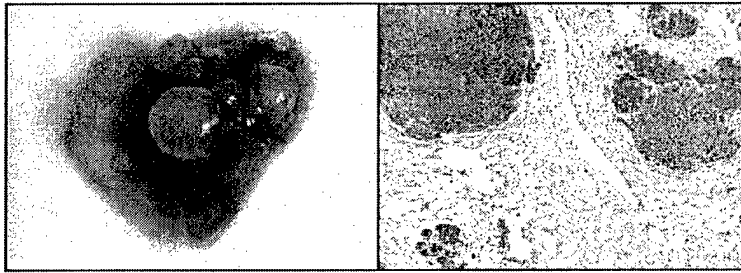


Fig. 8. Pulmonary metastases in MTB/TAN animals with primary mammary tumors. Left panel, gross photograph of a lung harvested from an MTB/TAN animal sacrificed while on doxycycline. Right panel, H&E-stained section of a lung with grossly visible nodules, confirming the presence of Neu-type adenocarcinomas embedded within the lung parenchyma.

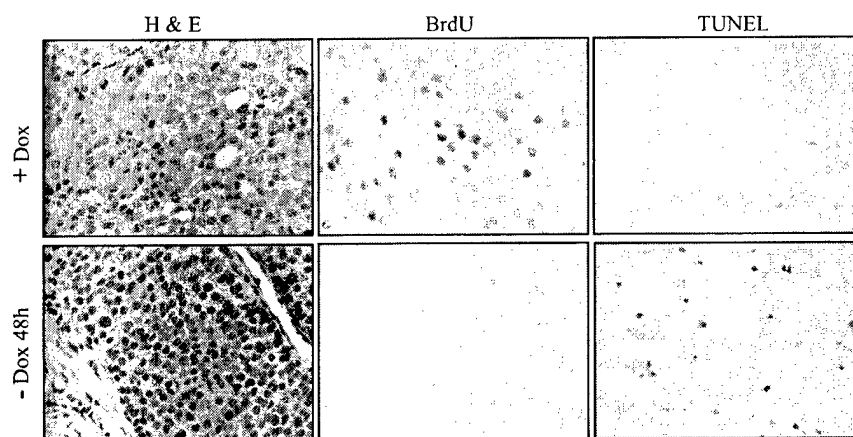


Fig. 9. Proliferation (BrdU) and apoptosis (TUNEL) rates in MTB/TAN animals with tumors on doxycycline (top panels) and 48 hours after doxycycline withdrawal (bottom panels).

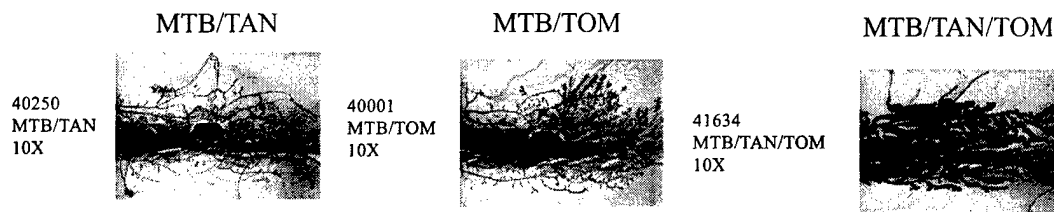


Fig. 10 Carmine-stained whole mounts of 14d-induced MTB/TAN, MTB/TOM, and MTB/TAN/TOM mammary glands.

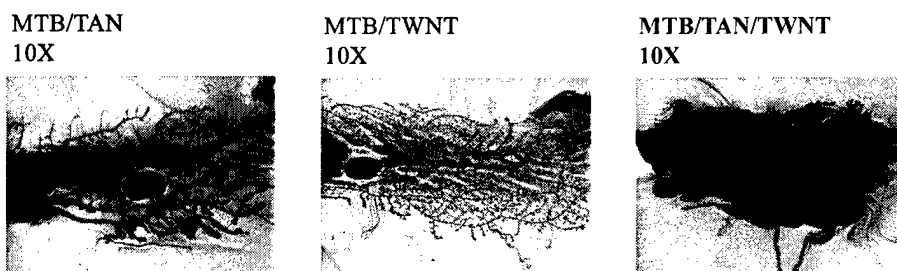


Fig. 11 Carmine-stained whole-mounts of 14d-induced MTB/TAN, MTB/TWNT, and MTB/TAN/TWNT mammary glands.

	MTB/TAN	MTB/TAN/TWNT	MTB/TAN/TOM
Dose (mg/ml)	0.1	0.1	0.1
Avg. (wk)	14.58	7.38	8.62
Stdev	3.6	3.18	1.74
SEM	0.87	0.80	0.46
N	17	13	14

Table 2. Mean tumor latencies in MTB/TAN, MTB/TAN/TWNT, and MTB/TAN/TOM mice chronically induced at 0.1 mg/ml doxycycline.

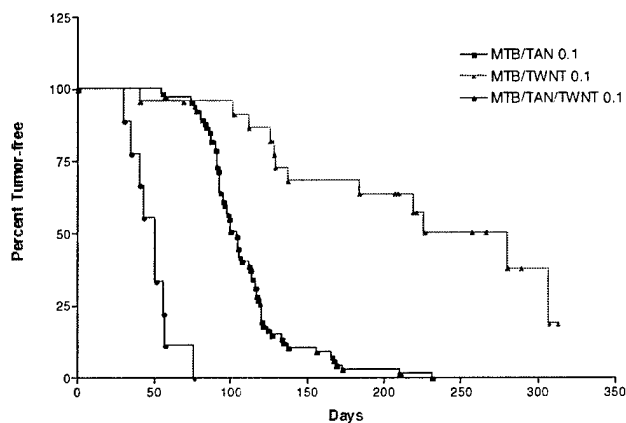


Fig 12. Tumor-free survival curves of MTB/TAN/TWNT (left-most line), MTB/TAN (center line), and MTB/TWNT (right-most line) mice chronically induced at 0.1 mg/ml doxycycline.

KEY RESEARCH ACCOMPLISHMENTS:

- Determined tumor incidence and latency in MTB/TAN and MTB/TWNT animals
- Determined titratability of MTB/TAN transgene expression and tumor latency
- Determined reversibility of Neu-induced mammary tumors
- Identified a decrease in proliferation and an increase in apoptosis in regressing Neu-induced tumors
- Observed a high rate of pulmonary metastases in MTB/TAN animals with primary mammary tumors

- Determined that Neu-induced pulmonary metastases remain dependent upon Neu transgene expression for maintenance of the transformed state.
- Determined that both Wnt and Myc synergize with Neu in mammary tumorigenesis in FVB mouse models.

REPORTABLE OUTCOMES:

Gunther EJ, Moody SE, Belka GK, Hahn KT, Innocent N, Dugan KD, Cardiff RD, Chodosh LA. Impact of p53 Loss on Reversal and Recurrence of Conditional Wnt-induced Tumorigenesis. *Genes Dev.* 2003 Feb; 17(4):488-501

Moody SE, Sarkisian CJ, Hahn KT, Gunther EJ, Pickup S, Dugan KD, Innocent N, Cardiff RD, Schnall MD, Chodosh LA. Conditional activation of Neu in the mammary epithelium of transgenic mice results in reversible pulmonary metastasis. *Cancer Cell* 2002 Dec; 2(6) 451-461.

D'Cruz CM, Gunther EJ, Boxer RB, Hartman JL, Sintasath L, Moody SE, Cox JD, Ha SI, Belka GK, Golant A, Cardiff RD, and Chodosh LA. c-MYC induces mammary tumorigenesis by means of a preferred pathway involving spontaneous Kras2 mutations. *Nat Med.* 2001 Feb;7(2):235-9.

CONCLUSIONS:

We have developed a model in which an activated form of Neu is conditionally expressed in the mammary epithelium of transgenic mice. We have found that these mice develop invasive mammary tumors with complete penetrance in a doxycycline dose-dependent manner. Furthermore, we have demonstrated that the vast majority of cells within these tumors remain dependent upon Neu expression for maintenance of the transformed state, as nearly all of the tumors regress fully upon doxycycline withdrawal. In addition, we have found that most animals maintained on doxycycline after the detection of the primary tumors ultimately develop pulmonary metastases, and that these also fully regress upon withdrawal of the Neu stimulus (Moody et al, 2003). We are currently focusing on investigating the nature of residual disease that may be present in regressed animals.

We have also investigated the interaction of the Neu oncogene with the oncogenes Wnt-1 and c-Myc. We have found that the simultaneous induction of Neu and Wnt-1 or of Neu and c-Myc results in a dramatic increase in mammary gland hyperplasia. This is followed by a significant reduction in tumor latency when compared to the induction of any of the three oncogenes alone. Future investigations are focusing on using microarray technology to investigate transcriptional changes that take place as a result of the induction of multiple oncogenes.

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Moody SE, Sarkisian CJ, Hahn KT, Gunther EJ, Pickup S, Dugan KD, Innocent N, Cardiff RD, Schnall MD, Chodosh LA. Conditional activation of Neu in the mammary epithelium of transgenic mice results in reversible pulmonary metastasis. *Cancer Cell* 2002 Dec; 2(6) 451-461.

Conditional activation of Neu in the mammary epithelium of transgenic mice results in reversible pulmonary metastasis

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Summary

To determine the impact of tumor progression on the reversibility of Neu-induced tumorigenesis, we have used the tetracycline regulatory system to conditionally express activated Neu in the mammary epithelium of transgenic mice. When induced with doxycycline, bitransgenic MMTV-rtTA/TetO-NeuNT mice develop multiple invasive mammary carcinomas, essentially all of which regress to a clinically undetectable state following transgene deinduction. This provides the first demonstration that Neu-initiated tumorigenesis is reversible. Strikingly, extensive lung metastases arising from Neu-induced mammary tumors also rapidly and fully regress following the abrogation of Neu expression. However, despite the near universal dependence of both primary tumors and metastases on Neu transgene expression, most animals bearing fully regressed Neu-induced tumors ultimately develop recurrent tumors that have progressed to a Neu-independent state.

Introduction

Breast cancer is the most common malignancy diagnosed among women in the United States and is the second leading cause of cancer mortality. While the efficacy of several chemotherapeutic approaches to this disease have been demonstrated, a major obstacle to the successful treatment of breast cancer has been the inability to effectively treat metastatic disease or prevent tumor recurrence, each of which ultimately leads to treatment failure and death.

Recently, novel therapeutic strategies have been developed that specifically target oncogenic pathways activated in subclasses of tumors. For example, amplification and overexpression of the protooncogene, *HER2/Neu*, occurs in 15%–30% of primary human breast cancers and is associated with aggressive tumor behavior, decreased time to relapse, and poor prognosis (Berger et al., 1988; Slamon et al., 1987). Clinical trials utilizing the neutralizing antibody Trastuzumab, which targets the *HER2/Neu* receptor tyrosine kinase, have demonstrated the

efficacy of this agent against *HER2/Neu*-amplified breast cancers even in advanced stages of this disease (Baselga et al., 1996; Cobleigh et al., 1999; Slamon et al., 2001; Vogel et al., 2002; Wang and Hung, 2001; Wang et al., 2001). However, while Trastuzumab—either alone or in combination with standard chemotherapeutic regimens—slows disease progression and improves survival, these cancers typically recur and become resistant to the therapeutic targeting of this pathway (Hortobagyi, 2001). The mechanism by which *HER2/Neu*-amplified breast tumor cells progress to a state that is independent of increased *HER2/neu* activity is unknown. As such, identifying secondary pathways that permit cancers to evade anti-*HER2/Neu* therapy is a critical next step in the development of more effective therapeutic approaches for this aggressive subset of tumors.

Relevant to this goal, mouse models of breast cancer initiated by defined oncogenic events relevant to breast cancer in humans have proven useful for investigating the process of tumorigenesis. For example, transgenic mice in which either wild-type *Neu* (*c-Neu*) or activated *Neu* (*NeuNT*) is constitutively

SIGNIFICANCE

The question of whether tumor progression impacts on the reversibility of oncogene-initiated events has important clinical implications for cancer therapy. Our data demonstrate that the vast majority of cells within even the most advanced stages of epithelial malignancy, namely metastases, remain dependent upon an initiating oncogenic event for maintenance of the transformed state. Nevertheless, we show that Neu-initiated mammary tumors commonly progress to a Neu-independent state and that the majority of animals bearing fully regressed tumors harbor residual neoplastic disease long after the apparently complete regression of their tumors. These findings challenge the assertion that all oncogene-initiated events are reversible and highlight the importance of determining the mechanisms by which tumor cells escape from their dependence on individual oncogenic pathways.

overexpressed in the mammary gland develop invasive mammary adenocarcinomas (Bouchard et al., 1989; Guy et al., 1992; Muller et al., 1988). However, the extent to which established Neu-induced tumors and metastatic lesions remain dependent upon this oncogenic signaling pathway for maintenance of the transformed state is unknown.

To address these questions, we have used the tetracycline regulatory system to conditionally express activated Neu in the mammary epithelium of transgenic mice. When induced with doxycycline, these mice develop multiple invasive mammary adenocarcinomas that metastasize to the lungs. Using this model, we show that targeted downregulation of the Neu pathway results in the rapid disappearance of essentially all Neu-initiated invasive primary tumors as well as pulmonary metastases. Our findings provide the first demonstration that, despite the multiple genetic events that are required for tumorigenesis and acquisition of the metastatic phenotype, even the most advanced stages of Neu-initiated malignancy remain dependent upon a single oncogenic mutation for growth and survival. However, consistent with the natural history of human cancers, we show that the majority of mice bearing fully regressed Neu-induced mammary tumors ultimately develop recurrences in the absence of Neu expression. This indicates that these animals harbor subpopulations of residual neoplastic cells that are eventually able to bypass their requirement for Neu to reestablish the malignant phenotype.

Results

Doxycycline-inducible expression of activated Neu

The c-Neu receptor tyrosine kinase is rendered constitutively active and oncogenic by a point mutation in its transmembrane domain (Bargmann et al., 1986). Mice capable of conditionally expressing this oncogenic form of Neu were generated using the tetracycline regulatory system by cloning the coding sequence of activated *Neu* (*NeuNT*) downstream of the minimal tet operator. Additionally, an IRES-firefly luciferase expression cassette was cloned downstream of activated *Neu* to serve as a surrogate reporter for transgene expression. Founder mice harboring this *TetO-NeuNT* transgene, referred to as TAN, were generated and mated to a previously described line of MMTV-rTA transgenic mice (MTB) to yield bitransgenic MTB/TAN offspring (D'Cruz et al., 2001; Gunther et al., 2002).

To determine whether this system would permit the conditional expression of activated Neu in a mammary-specific and doxycycline-dependent manner, transgene expression levels were determined by Northern hybridization analysis and measurement of luciferase activity (Figure 1). Analysis by these methods of mammary tissues harvested from female MTB/TAN mice following four days of doxycycline treatment revealed high levels of induction of activated *Neu* mRNA expression (Figure 1A). Similarly, luciferase activity in the mammary glands of induced MTB/TAN mice increased approximately 10,000-fold over baseline (Figure 1B). In contrast, transgene expression in the mammary glands of uninduced MTB/TAN animals as well as doxycycline-treated wild-type and MTB controls was undetectable either by Northern hybridization analysis or by analysis of luciferase activity (Figures 1A and 1B and data not shown).

Morphological examination of carmine-stained whole mounts revealed striking hyperplastic abnormalities in the mammary ductal trees of induced MTB/TAN mice following four days of

doxycycline induction (Figure 1C, left panels). Similarly, hematoxylin and eosin-stained sections from the mammary glands of induced MTB/TAN mice displayed extensive ductal hyperplasias and abnormal development, including the formation of multiple solid cellular masses along the primary ducts that resemble abortive side buds (Figure 1C, center and right panels). In contrast, tissue from uninduced MTB/TAN animals was morphologically and histologically indistinguishable from that of wild-type and MTB animals (Figure 1C and data not shown).

MTB/TAN animals develop invasive mammary carcinomas

Treatment of MTB/TAN animals with doxycycline for 21 days resulted in progressive hyperplastic changes that were particularly prominent in terminal end buds at the growing ends of ducts (Figure 2A, left panels). Terminal end buds were increased in size and surrounded by extensive clusters of acinar-like structures, many of which were separated by large blood vessels (Figure 2A, center and right panels, and data not shown). These histological changes are typical of those induced by the ErbB2 signaling pathway (Cardiff et al., 2000; Cardiff and Muller, 1993). Mammary glands from doxycycline-treated MTB and TAN controls and from MTB/TAN animals maintained in the absence of doxycycline for more than a year did not display any morphological abnormalities (Figure 2A and data not shown).

Consistent with the profound hyperplastic changes noted above, chronic induction of *NeuNT* transgene expression in MTB/TAN animals with 2 mg/ml doxycycline resulted in the rapid development of multiple mammary tumors with 100% penetrance and a latency of 6 weeks (Figure 2B). Tumors arose stochastically and were focal in nature, although essentially all mammary glands in chronically induced MTB/TAN animals harbored tumors. No tumors were observed in uninduced MTB/TAN animals or in doxycycline-treated MTB and TAN controls over periods exceeding one year (Figure 2B).

Histological analysis of mammary tumors arising in doxycycline-induced MTB/TAN mice revealed invasive solid nodular carcinomas typical of Neu/ErbB2-initiated mammary tumors (Cardiff et al., 2000; Cardiff and Muller, 1993; Muller et al., 1988). Tumor cells possessed bland, uniform oval nuclei, abundant pink-red cytoplasm, and lacked significant glandular differentiation as is typical of the "intermediate cells" of Neu/ErbB2 tumors (Figure 2C, left panel). Small nests of intermediate cells were subdivided by a rich microvasculature. An additional phenotype, which has not been previously described in Neu/ErbB2 transgenics, was identified in several tumors consisting of nests and cords of nodular cells with bright red cytoplasm invading a dense, vascular stroma (Figure 2C, center panel). These tumors resemble the most common type of human breast cancers that are currently classified as "breast cancer, no specific type" (Cardiff and Wellings, 1999; Maglione et al., 2001). Immunohistochemical analysis using anti-Neu and anti-Cytokeratin 8 antibodies confirmed that all Neu-initiated tumors express high levels of Neu protein and are of luminal epithelial origin (Figure 2C, right panel and data not shown).

Invasive mammary carcinomas require Neu for tumor maintenance

The focal nature and stochastic appearance of mammary tumors in MTB/TAN mice suggested that additional genetic alterations were likely to be required for Neu-induced tumorigenesis. We wished to determine whether invasive mammary carcinomas

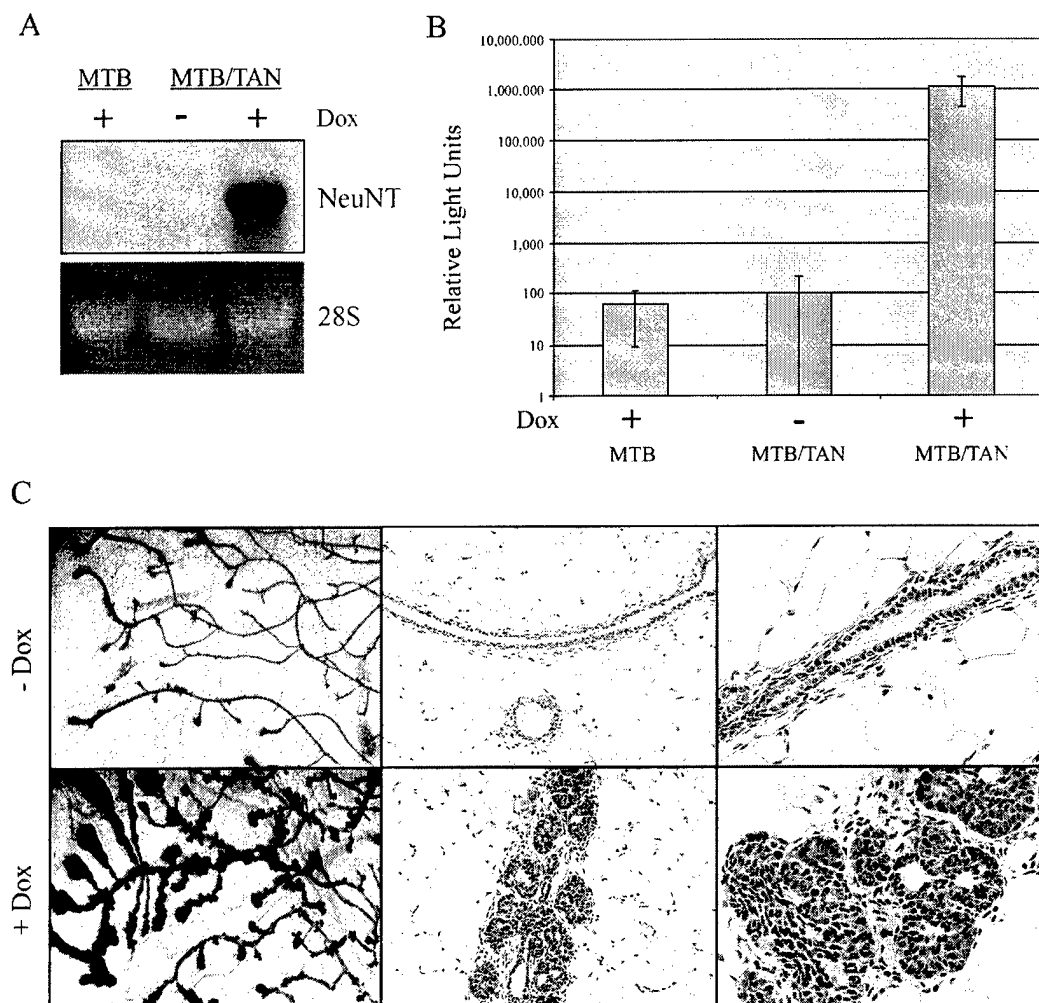


Figure 1. Doxycycline-dependent expression of NeuNT results in abnormal alveolar development

A: Northern analysis of total RNA from mammary glands of 6.5 week MTB and MTB/TAN animals maintained on doxycycline for 4 days. The blot was probed with a DNA fragment specific for Neu. 28S rRNA is shown as a loading control.

B: Luciferase activity assays performed on mammary tissue from 6.5 week MTB and MTB/TAN animals maintained on doxycycline for 4 days. Assays were performed in triplicate and relative light units (RLU) were normalized to total protein levels.

C: Carmine-stained whole mounts (left panels, magnification 25 \times) and hematoxylin and eosin (H&E)-stained sections of mammary glands (middle and right panels, magnification 200 \times and 400 \times) from 6.5 week MTB/TAN animals administered doxycycline for 4 days and uninduced MTB/TAN controls.

arising in MTB/TAN mice remained dependent upon Neu for maintenance of the transformed state or, alternately, whether secondary mutations that occur during the process of tumorigenesis render Neu dispensable for tumor growth and survival.

To address this question, doxycycline was withdrawn from a cohort of chronically induced tumor-bearing MTB/TAN animals, each of which harbored multiple palpable mammary tumors ranging in size from 25 to 360 mm². Northern hybridization analysis demonstrated that *NeuNT* transgene expression was rapidly downregulated in primary tumors within 24 hr following doxycycline withdrawal and was undetectable within 48 hr (Figure 3A). Strikingly, 44 of 47 tumors (94%) rapidly and fully regressed to a nonpalpable state following doxycycline withdrawal with a mean time to complete regression of 17 \pm 12 days (Figures 3B and 3C). Of the three tumors that failed to regress completely, one was determined at the time of sacrifice

to be a hemorrhagic cyst, whereas two tumors regressed partially and then resumed growth. Neither of these expressed detectable levels of the *NeuNT* transgene (data not shown). These findings demonstrate that the vast majority of epithelial cells within Neu-induced tumors remain dependent upon Neu for maintenance of the transformed state.

To analyze the cellular mechanism of regression in Neu-induced mammary tumors, doxycycline was withdrawn from an independent set of MTB/TAN mice harboring tumors and animals were sacrificed 48 hr later following labeling with BrdU. Anti-BrdU immunohistochemistry revealed that cellular proliferation rates were dramatically downregulated in Neu-induced tumors within 48 hr following doxycycline withdrawal (Figure 3D, center panels). Conversely, whereas apoptotic cells were rarely detected in tumors harvested from mice maintained on doxycycline, marked increases in the numbers of TUNEL-posi-

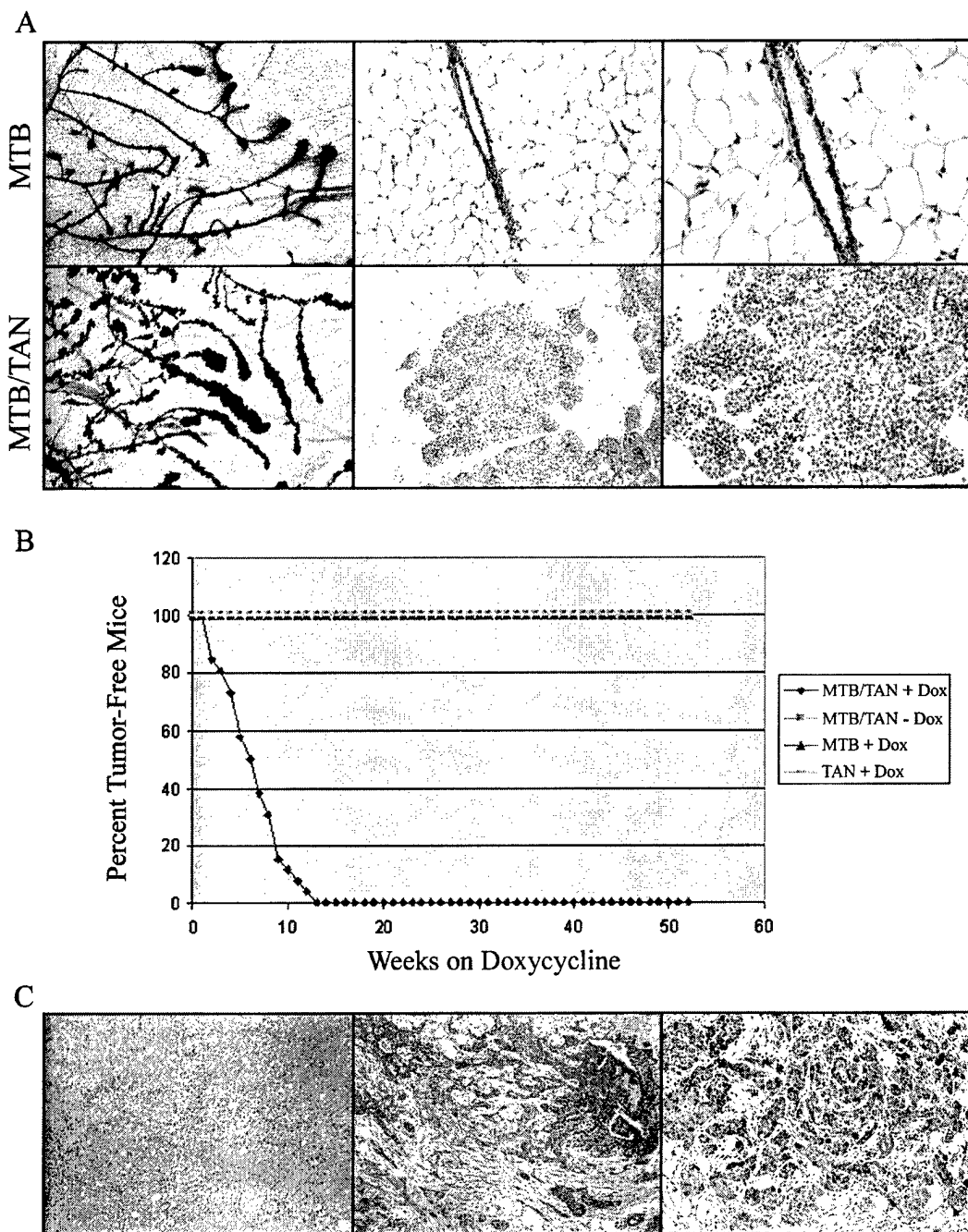


Figure 2. MTB/TAN mice maintained chronically on doxycycline develop mammary adenocarcinomas

A: Carmine-stained whole mounts (left panels, magnification 50 \times) and H&E-stained sections (center and right panels, magnification 200 \times and 800 \times) of mammary glands from 9 week MTB/TAN mice maintained on doxycycline for 21 days.

B: Tumor-free survival curve for MTB/TAN ($n = 26$), TAN ($n = 8$), and MTB ($n = 10$) mice chronically administered 2 mg/ml doxycycline, and MTB/TAN ($n = 13$) mice maintained off doxycycline.

C: H&E-stained sections from tumors with characteristic Neu phenotype (left panel) and atypical Neu phenotype (middle panel) and anti-Neu immunohistochemistry (right panel) performed on a tumor arising in an MTB/TAN animal. Magnification 200 \times .

tive cells were observed in tumors harvested from animals 48 hr following doxycycline withdrawal (Figure 3D, right panels). These results suggest that the initial phase of tumor regression in MTB/TAN animals induced by *Neu* transgene downregulation is due to decreased proliferation and increased apoptosis.

Pulmonary metastases remain dependent upon Neu

A large percentage of MTB/TAN animals bearing primary mammary tumors eventually develop a distinctive ill phenotype characterized by hunched posture, ruffled fur and labored breathing that is uniformly fatal within one week. Examination of the lungs

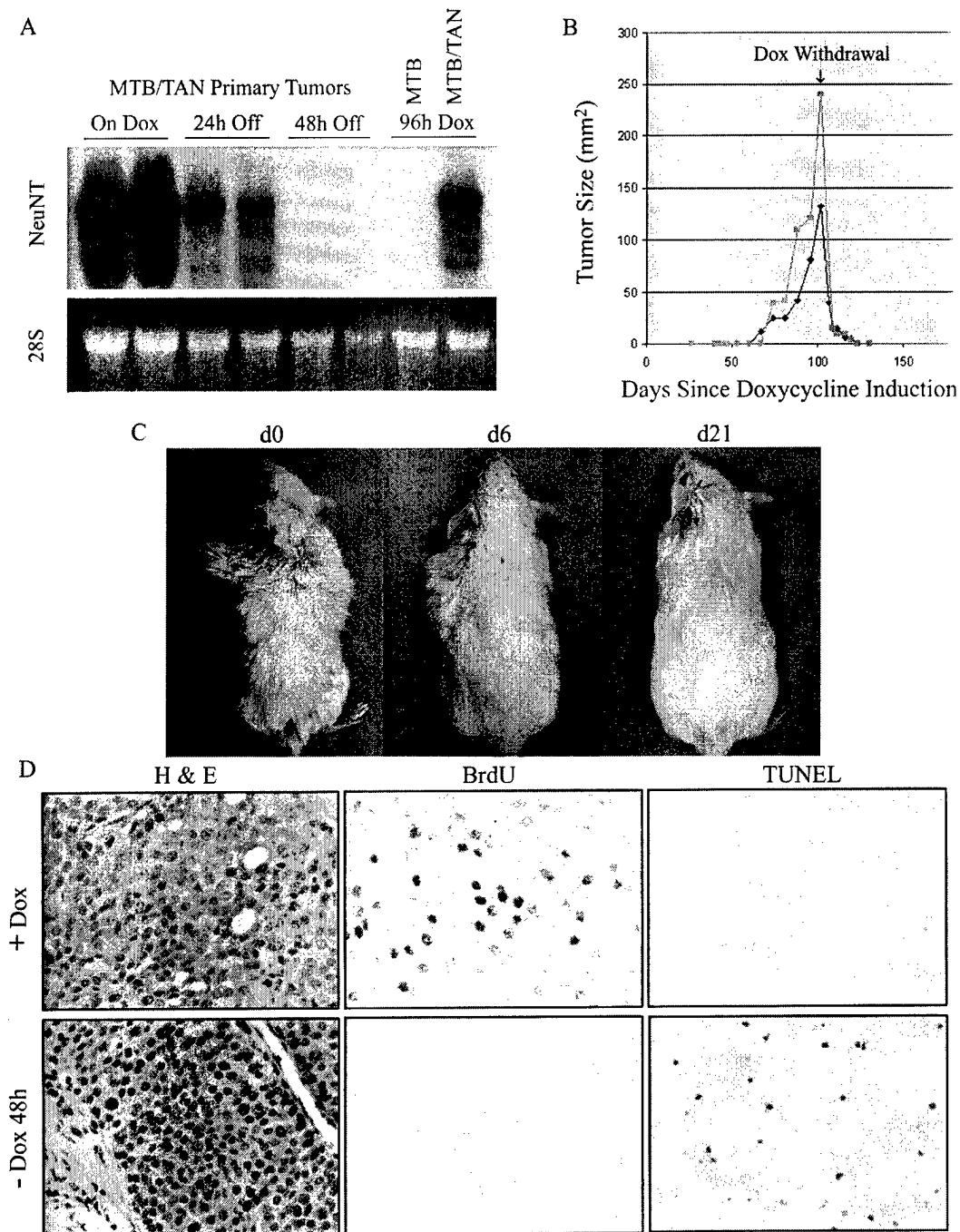


Figure 3. NeuNT-induced tumors regress fully following withdrawal of the NeuNT stimulus

A: Northern analysis of total RNA from MTB/TAN tumors harvested in the presence of doxycycline and 24 or 48 hr after doxycycline withdrawal. The blot was probed for Neu. Mammary gland samples harvested from MTB and MTB/TAN animals maintained on doxycycline for 96 hr are shown as controls. 28S rRNA is shown as a loading control.

B: Representative graphs depicting the growth and regression of two tumors in a mouse from which doxycycline was withdrawn. Doxycycline was removed on day 102.

C: Photographs of a chronically induced MTB/TAN animal at the time of doxycycline withdrawal (d0) and on the 6th (d6) or 21st day (d21) following doxycycline withdrawal.

D: H&E-stained sections, anti-BrdU immunohistochemistry, and TUNEL analysis of MTB/TAN tumors harvested in the presence of doxycycline and 48 hr following doxycycline withdrawal. Magnification 800 \times .

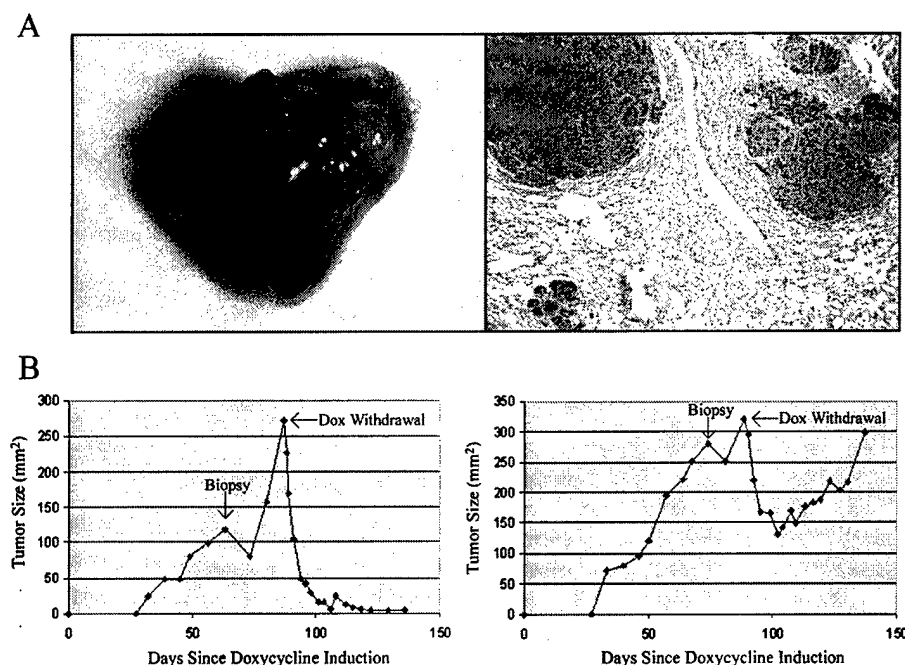


Figure 4. Grafted pulmonary metastases from tumor-bearing MTB/TAN mice are reversible

A: Gross pathology (3.2×) and H&E-stained section (100×) of lungs containing pulmonary metastases harvested from chronically induced MTB/TAN animals bearing primary mammary tumors.

B: Representative graphs displaying growth on doxycycline and regression following doxycycline withdrawal leading either to complete (top panel) or to partial (bottom panel) regression of pulmonary metastasis grafts implanted on the flanks of syngeneic hosts.

from 26 animals exhibiting this phenotype revealed that 24 (92%) harbored grossly visible solid pulmonary nodules on the pleural surface (Figure 4A, left panel). Microscopic analysis confirmed that these nodules were pulmonary metastases with histological features characteristic of Neu-induced mammary epithelial carcinomas (Figure 4A, right panel). Luciferase activity levels in lungs bearing metastatic lesions were comparable to levels detected in the primary mammary tumors (data not shown).

Despite our observation that NeuNT-induced primary mammary tumors remain dependent upon Neu expression for maintenance of the transformed state, we considered the possibility that the same genetic alterations that confer the aggressive growth properties required for Neu-induced mammary tumor cells to metastasize might also facilitate the progression of tumor cells to a Neu-independent state. To address this question, doxycycline treatment was withdrawn from a cohort of chronically induced, tumor-bearing MTB/TAN animals that had developed the characteristic respiratory phenotype described above associated with pulmonary metastases. Whereas this condition is uniformly fatal in animals maintained on doxycycline, all 9 tumor-bearing mice displaying this phenotype at the time of doxycycline withdrawal rapidly recovered and resumed a normal respiratory rate. Given the high degree of correlation between the respiratory phenotype observed in tumor-bearing MTB/TAN animals and the presence of pulmonary metastases, our observations suggested the possibility that metastases in MTB/TAN animals might also remain dependent on Neu for growth.

To pursue this hypothesis, pulmonary metastases and primary tumors from chronically induced MTB/TAN mice were grafted onto the flanks of syngeneic hosts maintained on doxycycline. Following the outgrowth of these grafts, doxycycline was withdrawn from graft recipients. 68% (13/19) of tumor grafts from pulmonary metastases and a similar fraction (57%; 8/14) of grafts from primary mammary tumors regressed to a nonpal-

pable state following doxycycline withdrawal (Table 1). Five grafts in each group regressed only partially and then resumed growth, whereas one graft in each group regressed partially, but did not resume growth (Table 1 and Figure 4B). Of note, none of the grafts that resumed growth expressed the *NeuNT* transgene or detectable levels of endogenous *ErbB2* (data not shown). These observations demonstrate that the majority of grafted pulmonary metastases in MTB/TAN mice remain dependent upon Neu for maintenance of the transformed state.

The apparent discrepancy between the fractions of primary mammary tumors and grafted primary mammary tumors that regressed fully following doxycycline withdrawal (94% versus 57%, respectively) suggested that genetic alterations that render tumor cells independent of Neu might occur during the outgrowth of tumor grafts, but not during the process of tumorigenesis in situ. This, along with the consistent resolution of respiratory symptoms noted following doxycycline withdrawal from tumor-bearing mice, suggested that an even greater fraction of pulmonary metastases in situ may remain dependent on *NeuNT* transgene expression for maintenance of the transformed state.

To test this hypothesis directly, magnetic resonance imaging was performed on tumor-bearing MTB/TAN mice that had developed the characteristic respiratory phenotype associated with

Table 1. ■■■■

	Primary tumors	Primary tumor grafts	Metastasis grafts
Fully regressed	44/47 (94%)	8/14 (57%)	13/19 (68%)
Partially regressed, resumed growth	2/47 (4%)	5/14 (36%)	5/19 (26%)
Partially regressed, no regrowth	1/47 (2%)	1/14 (7%)	1/19 (5%)

pulmonary metastases. These images confirmed the presence of widespread pulmonary nodules (Figure 5A, middle column). As before, withdrawal of doxycycline from these animals resulted in the regression of primary mammary tumors as well as the resolution of their respiratory phenotype. Reimaging these animals by MRI 30 days later revealed that nodules were no longer detectable in the lungs (Figure 5A, right column). Moreover, histological analysis of the lungs from these animals confirmed the absence of neoplastic disease (Figure 5B).

In aggregate, the high rate of metastasis in tumor-bearing MTB/TAN animals displaying a respiratory phenotype, the reproducible resolution of this phenotype in animals taken off doxycycline, the regression of grafted pulmonary metastases, and the regression of pulmonary metastases *in vivo* all suggest that the vast majority of cells within most, if not all, pulmonary metastases arising from Neu-induced mammary tumors remain dependent upon continued Neu expression for maintenance of the transformed state.

Fully regressed tumor animals recur in MTB/TAN in the absence of doxycycline

The complete regression of Neu-induced invasive mammary adenocarcinomas and metastases that we observed in MTB/TAN mice was unexpected, since clinical experience indicates that human epithelial malignancies are rarely, if ever, cured by treatment with a single agent. Rather, we anticipated that at least some cells in Neu-induced tumors would have acquired the ability to grow in a Neu-independent manner. This fact prompted us to examine MTB/TAN mice bearing fully regressed tumors for evidence of tumor recurrence. Eleven MTB/TAN mice in which doxycycline withdrawal had led to the complete regression of all tumors were maintained off doxycycline and monitored for tumor recurrences. A total of 8 tumors recurred in 7 animals after an average of 153 ± 93 days (range 27–300 days) off doxycycline (Figure 6A). That these tumors represent recurrences of Neu-initiated tumors rather than the *de novo* formation of tumors in the absence of doxycycline is strongly suggested by our repeated observation that uninduced MTB/TAN animals do not develop tumors, even over periods exceeding 18 months ($n = 13$). Northern analysis failed to detect *NeuNT* transgene expression in any of these recurrent tumors, indicating that tumor recurrences are not merely a consequence of transgene activation in the absence of doxycycline (Figure 6B). Similarly, Northern analysis as well as anti-ErbB2 immunohistochemistry failed to detect upregulation of endogenous *ErbB2* in recurrent tumors (data not shown).

Together, these findings strongly suggest that a subset of cells within Neu-initiated tumors progress to a state that is independent of Neu overexpression for survival and growth. More importantly, our observations demonstrate that despite the near universal regression of Neu-initiated primary mammary tumors to a nonpalpable state, the majority of fully regressed tumor-bearing animals in this system harbor residual neoplastic disease for periods of up to a year following the clinical disappearance of their tumors.

Discussion

The evolution of human cancers is characterized by the progressive selection and outgrowth of mutant clones that possess increasingly aggressive properties, such as loss of hormone

dependence, resistance to chemotherapeutic agents, and the ability to invade tissues and metastasize. This process of tumor progression is ultimately responsible for cancer mortality. While the reversibility of several oncogene-induced primary tumors has been demonstrated using conditional transgenic mouse models, the impact of tumor progression on oncogene reversibility has not been addressed in any system to date.

We have developed a novel inducible transgenic mouse model for HER2/Neu-overexpressing breast cancers that displays many features of human tumor progression, including invasion, metastasis, and recurrence. Given the highly aggressive nature of both human and murine breast cancers overexpressing HER2/Neu, we considered the possibility that Neu-induced mammary carcinomas would continue to grow despite downregulation of the initiating oncogenic stimulus. In contrast, we found that essentially all primary tumors induced by Neu regress to a clinically undetectable state following transgene deinduction by doxycycline withdrawal. Thus, despite the multiple genetic and epigenetic alterations that occur during Neu-induced mammary tumorigenesis, the vast majority of cells within these tumors remain dependent upon Neu for maintenance of the transformed state. Surprisingly, we have also found that extensive lung metastases arising in tumor-bearing animals also rapidly and fully regress following the abrogation of Neu expression. However, despite the reversibility of Neu-induced primary tumors and metastases, we find that most if not all animals in which Neu-induced mammary carcinomas have regressed to a clinically undetectable state still harbor residual neoplastic disease long after the clinical disappearance of their tumors. Moreover, these residual cells ultimately give rise to recurrent tumors that grow in a Neu-independent manner, strongly suggesting that subpopulations of neoplastic mammary epithelial cells are able to escape their requirement for Neu to reestablish a malignant phenotype. These findings contradict the assertion that all oncogene-initiated events are reversible.

The dependence of tumors on a single oncogene has previously been demonstrated in conditional transgenic mouse models for H-ras-induced melanomas, K-ras-induced adenocarcinomas of the lung, BCR-ABL-induced leukemias, and Myc-induced leukemias, lymphomas, and islet cell tumors (Chin et al., 1999; Felsher and Bishop, 1999; Huettner et al., 2000; Fisher et al., 2001; Pelengaris et al., 2002). This has led to the frequent assumption that tumors induced by other oncogenes in other tissues will similarly remain dependent upon the initiating oncogene for maintenance of the transformed state. However, current evidence challenges this view and suggests instead that dependence upon an initiating oncogenic event is not a monolithic property of tumors. For example, in marked contrast to the near total reversibility of MYC-induced neoplasia reported in the hematopoietic compartment and in pancreatic β cells, we have previously observed that the majority of tumors induced by MYC in the mammary gland do not remain dependent upon MYC for maintenance of the transformed state (D'Cruz et al., 2001). In turn, the tendency of MYC to induce mammary tumors that are MYC-independent contrasts sharply with our current findings regarding the near universal dependence of Neu-induced mammary tumors on Neu. These observations emphasize the importance of investigating the reversibility of different oncogenes and different tumor types individually. Given that we have analyzed both *Neu* and *c-MYC*-initiated mammary tu-

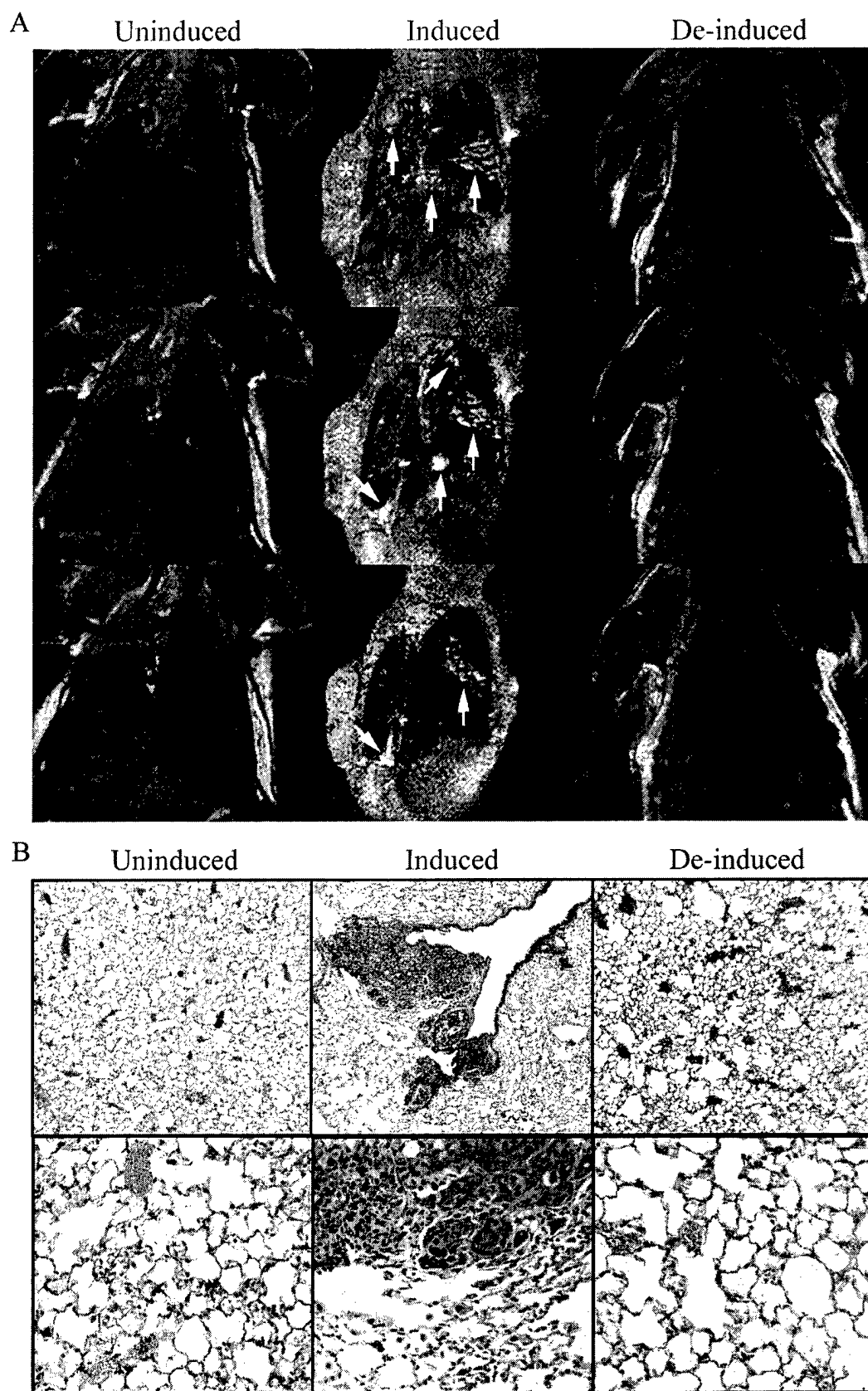


Figure 5. MRI and histological analysis of the reversibility of NeuNT-induced pulmonary metastases in situ

A: Magnetic resonance images of lungs from an uninduced MTB/TAN animal (Uninduced, left column of panels), an MTB/TAN tumor-bearing animal on doxycycline with the characteristic respiratory phenotype associated with pulmonary metastases (Induced, middle column of panels), and the same

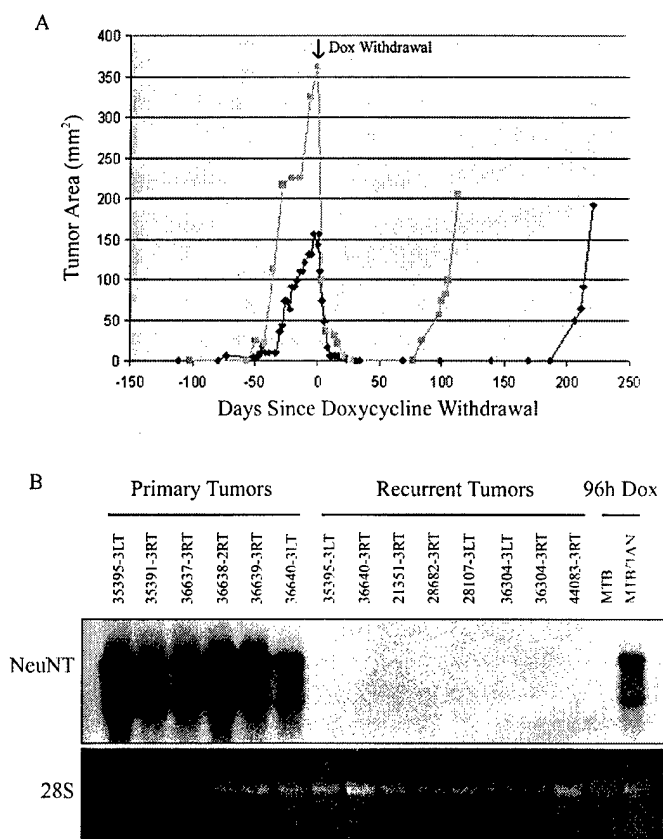


Figure 6. MTB/TAN mice harboring fully regressed tumors develop Neu-independent tumor recurrences

A: Representative graphs of MTB/TAN tumors displaying growth on doxycycline, full regression following doxycycline withdrawal, and recurrence in the absence of doxycycline.

B: Northern analysis of total RNA from primary and recurrent tumors harvested from MTB/TAN animals. Primary tumor samples were obtained by biopsy of tumors in animals maintained on doxycycline. Recurrent tumors arose following doxycycline withdrawal and full regression of the primary tumor to a nonpalpable size. The blot was probed with a DNA fragment specific for Neu. 28S rRNA is shown as a loading control.

are epithelial, investigating the phenomenon of oncogene-reversibility in epithelial cell types in which oncogenes relevant to the corresponding human cancers have been activated has obvious clinical importance.

The potential impact of tumor progression on the reversibility of oncogene-initiated events is an important issue that has critical clinical implications. In this regard, the dramatic regression of invasive primary tumors that we observed following transgene deinduction raised the question of whether distant metastases would remain similarly dependent upon an initiating genetic lesion for maintenance and growth. To be sure, the observation that primary tumors are reversible does not imply that metastases arising from these tumors will also be reversible. Indeed, it might reasonably be predicted that the same adaptations that confer the aggressive growth properties required for Neu-induced mammary tumor cells to metastasize might also facilitate Neu-independent tumor growth and maintenance. If so, one would predict that metastatic lesions arising from Neu-induced tumors would be more resistant to the effects of down-regulating Neu expression than cells from the primary mammary tumors. In contrast to this expectation, we have demonstrated that extensive lung metastases arising in animals bearing Neu-induced mammary tumors rapidly and fully regress following the abrogation of Neu expression. Our results demonstrate that despite the acquisition of mutations that render tumor cells able to invade the vasculature, disseminate, survive, and establish growth at distant sites, the vast majority of tumor cells within even the most advanced stages of malignancy remain dependent upon a single genetic mutation for growth and maintenance of the transformed phenotype. Thus, in contrast to the general presumption that the same genetic events that contribute to tumor progression also render metastatic lesions less responsive to pharmacologic intervention, our findings indicate that advanced tumor stage per se does not render malignant cells independent of Neu signaling for their maintenance.

Recent findings from a number of inducible transgenic mouse models of cancer have been interpreted to suggest the possibility that many oncogene-initiated events may be reversible and that human tumors may be similarly reversible with targeted therapy. Indeed, our finding that reversing a single oncogenic mutation results in the complete regression of both primary tumors and metastatic disease is consistent with this notion. However, since metastatic epithelial cancers are typically incurable, even with the use of combinatorial therapies, the complete reversibility of oncogene-initiated events is clearly inconsistent with clinical experience. In fact, we find that despite the near universal regression of Neu-initiated primary mammary tumors to a nonpalpable state, the majority of mice bearing fully regressed mammary tumors harbor residual foci of viable neoplastic cells even after periods of up to a year following the clinical disappearance of their tumors. Moreover, these foci of residual disease ultimately lead to the emergence of recurrent

morigenesis in the same tissue compartment, in an identical genetic background, and using the same MMTV-rtTA transactivator line, our findings imply that the likelihood of developing a transgene-independent tumor is an intrinsic property of the initiating oncogenic event. That is, different oncogenic pathways are likely to have different probabilities of being rendered dispensable for tumor maintenance and growth, and the propensity of cancers to become oncogene-independent is therefore almost certainly a function both of the oncogene that is activated and of the cellular compartment in which oncogene activation occurs. Consequently, since the vast majority of human cancers

animal as that imaged in the middle column 30 days after withdrawal of doxycycline (Deinduced, right column of panels). Each column of panels represents three coronal sections, progressing anterior to posterior, taken at equivalent levels from each animal. Focal areas of metastasis are indicated by arrows. The primary tumor is denoted by an asterisk.

B: H&E-stained lung sections from an uninduced MTB/TAN animal (Uninduced), an MTB/TAN animal sacrificed while on doxycycline that harbored grossly visible pulmonary metastases (Induced), and the Deinduced MTB/TAN animal imaged in **A** from which doxycycline had been withdrawn (Deinduced). Magnification 50× (upper row) and 200× (lower row).

tumors that grow in a Neu-independent manner. Whether or not these tumors have escaped their dependence on Neu by activating downstream components of the Neu pathway remains to be investigated. In either case, however, our observation that residual Neu-induced tumor cells typically progress to a Neu-independent state mirrors the propensity of human tumor cells to progress to more advanced stages of malignancy and demonstrates that all oncogene-initiated events are not reversible. These findings contrast with the apparent lack of progression to oncogene-independent state in several other conditional transgenic models for cancer.

Recently, Jain et al. demonstrated that even brief inactivation of MYC in osteogenic sarcoma cells results in the sustained regression of these tumors and, furthermore, that MYC transgene deinduction protects these mesenchymal cells from subsequent MYC-induced tumorigenesis (Jain et al., 2002). In contrast to this observation, we find that even prolonged inactivation of Neu fails to yield sustained regression of Neu-induced mammary tumors, as these tumors instead commonly recur in a Neu-independent manner. As such, the phenomenon described by Jain et al. for MYC in transplanted osteogenic sarcoma cells does not appear to apply to Neu in intact mammary adenocarcinomas. Given the important biological role for Neu/HER2 in human breast cancers, and the availability and increasing use of an anti-HER2 therapy in the clinic, our observations suggest that attempts to extrapolate the findings of Jain et al. to human epithelial malignancies should be made cautiously.

Finally, the importance of developing chemotherapeutic strategies that target multiple pathways in epithelial cancers is highlighted by clinical experience as well as by our observation that Neu-induced mammary tumors typically progress to a Neu-independent state. Since an increasing number of therapies targeting molecular pathways known to play a role in cancer are either in clinical use or are under development, elucidation of the mechanisms by which tumor cells escape their dependence on these targeted pathways represents a critical next step in cancer research. Such knowledge will facilitate the development of more effective therapeutic approaches that achieve tumor cell eradication by targeting both primary oncogenic pathways as well as secondary pathways of tumor escape. By permitting the temporal dissection of tumor initiation, establishment, progression, metastasis, and recurrence, we believe that this model system provides a valuable new opportunity for examining the molecular events that contribute to the progression of Neu-induced mammary carcinomas as well as for analyzing the nature of residual neoplastic disease.

Experimental procedures

Animals and tissues

TetO-NeuNT mice were engineered by cloning the coding sequence of activated Neu (a gift of William Muller) downstream of the tet operator in pTet-Splice (Gibco-BRL). An IRES-Firefly Luciferase sequence was cloned downstream of NeuNT using the IRES from MIGR1 (a gift of Warren Pear) and Luciferase from pGL3-Basic (Promega). Founder lines were generated by injecting the linearized construct into fertilized oocytes harvested from super-ovulated FVB mice.

Transgenic mice were housed under barrier conditions with a 12 hr light/dark cycle and access to food and water ad libitum. Induced animals were administered doxycycline (0.1–2 mg/ml) (Sigma) in their drinking water, which was replaced weekly. Animals were inspected for tumors, and existing tumors were measured weekly. At the indicated times of sacrifice, animals were killed by CO₂ asphyxiation and tissues were either snap-frozen on dry ice for protein or RNA analysis, or fixed in 4% paraformaldehyde for morphological and immunohistochemical analysis.

Luciferase assays

Snap-frozen mammary gland tissue was analyzed using the Luciferase Assay System (Promega) per manufacturer's instructions. Tissue was dounced in 100 μ l 1 \times Reporter Lysis Buffer and lysates were centrifuged at 4°C for 1 min at 14,000 rpm. 20 μ l of lysate was injected with Luciferase Assay Substrate and activity was read in a Monolight 2010 luminometer. Luciferase activity levels were normalized to total protein levels as determined by Lowry Protein Assay (BioRad).

Whole mounts and histology

Number 3 or 4 mammary glands were mounted on glass slides, fixed overnight in 4% paraformaldehyde, and transferred to 70% ethanol. For whole mounts, glands were rinsed in water for 5 min and stained in a filtered solution of 0.2% carmine (Sigma) and 0.5% aluminum potassium sulfate for 1–3 days. Glands were then dehydrated sequentially through 70%, 90%, and 100% ethanol for 15 min each, then defatted and stored in methyl salicylate. For histological analysis, fixed glands were blocked in paraffin, sectioned, and stained with hematoxylin and eosin.

Immunohistochemistry

For BrdU analysis, animals were injected with 0.05 mg BrdU per gram body weight two hours prior to sacrifice. The number four mammary gland was harvested and fixed overnight in 4% paraformaldehyde, transferred to 70% ETOH, and embedded in paraffin. 5 μ m sections on ProbeOn Plus (Fisher) slides were dewaxed in xylene, then sequentially rehydrated in 100%, 95%, and 70% ETOH, followed by phosphate buffered saline (PBS). Sections were pretreated in 2N HCl for 20 min at RT, washed in 0.1 M Borate buffer (pH 8.5) \times 2, and rinsed in PBS. BrdU immunohistochemistry was performed using the Vectastain Elite ABC Kit (Vector Laboratories), rat anti-BrdU IgG (Vector), and a secondary biotinylated rabbit anti-rat IgG antibody according to manufacturer's instructions. Sections were counterstained for 10 min in 0.5% (w/v) methyl green in 1.0 M NaOAc (pH 4.0).

TUNEL analysis was performed using the Apoptag Peroxidase Kit (Intergen) according to manufacturer's instructions. Sections were pretreated in Proteinase K (20 μ g/ml) for 15 min at RT, washed in deionized water twice for 2 min each, incubated in equilibration buffer, then incubated at 37°C for 1 hr with a 1:10 Dilution of TdT Enzyme in 1 \times reaction buffer. Reactions were terminated, developed using anti-digoxigenin-Alkaline Phosphatase Fab fragments (BMB) and nitroblue tetrazolium chloride per manufacturer's instructions, and counterstained in methyl green.

For Neu/ErbB2 IHC, paraffin-embedded tumors were sectioned at 5 μ m and antigen retrieval was accomplished by microwaving in citrate buffer. Anti-ErbB2 antibody PH511.xs (Binding Site) was detected using the Vector ABC kit. Images were captured using a Kontron camera model 8102 on an Olympus BH2 microscope, digitized using Photoshop 6.0 with the Kontron ProgRes plugin module, color enhanced, and balanced for contrast.

Northern analysis

Snap-frozen tissue was homogenized in guanidine thiocyanate supplemented with 7 μ l/ml 2-mercaptoethanol, and RNA isolated by centrifugation through cesium chloride as previously described (Rajan et al., 1996). Total RNA (3 μ g per blot) was separated on a 1% LE agarose gel, and passively transferred to Gene Screen (NEN). Northern hybridization was performed per manufacturer's instructions using PerfectHyb Plus Hybridization Buffer (Sigma) and a ³²P-labeled cDNA probe spanning the 3' end of the Neu coding sequence and the 5' end of the IRES.

Tumor grafting

Chronically induced tumor-bearing MTB/TAN animals that had developed a characteristic phenotype of ruffled fur and labored breathing were sacrificed. Primary tumors and grossly visible pulmonary metastases were harvested and chilled on ice in DMEM (Cellgro) prior to being grafted subcutaneously onto the flanks of anesthetized recipient animals. Recipient animals were then placed on doxycycline treatment, and graft outgrowths were biopsied when they reached a size of approximately 15 \times 15 mm². Grafted animals were maintained on doxycycline after biopsy to document continued graft growth, at which time doxycycline was withdrawn and the regression behavior of the grafts was monitored.

Magnetic resonance imaging

Animals were lightly anesthetized using a 1% isoflurane air mixture. Subdermal needle electrodes were placed in the two forelegs, a thermistor was placed rectally, and animals were mounted in a home-built 5 × 9 cm linearly polarized birdcage coil. All MR imaging was performed on a 4.7T horizontal bore INOVA spectrometer (Varian, Palo Alto, CA) equipped with 12 cm 25 g/cm gradients. ECG, respiration, and core body temperature were monitored using a prototype MR compatible small animal monitoring device (SA Instruments, Bayshore, NY). This device also generated the signals for gating the spectrometer and regulating a warm air source used to maintain body temperature. Combined respiratory and cardiac gating was used in all studies in order to minimize motion artifacts. Images were generated using the standard spin echo sequence with TE/TR = 15/250 msec, a slice thickness of 1 mm, a field of view of 6 × 3 cm, and a matrix of 256 × 128.

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